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ORIGINAL PAPER

Exogenous application of thiamin promotes growth and antioxidative defense system at initial phases of development in salt-stressed plants of two maize cultivars differing in salinity tolerance

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Abstract The effects of thiamin (Thi) applied as seed soaking or foliar spray on some key physiological parameters were investigated in two differentially salt-responsive maize (*Zea mays* L.) cultivars, DK 5783 and Apex 836 F1, exposed to saline stress in two different experiments. An initial experiment (germination experiment) was designed to identify appropriate doses of Thi which could lessen the deleterious effects of salt on plants and screen all available maize cultivars for their differential tolerance to salt stress (100 mM NaCl). The seeds of nine maize cultivars were soaked for 24 h in solutions containing six levels of Thi (25, 50, 75, 100, 125 and 150 mg l^{-1}). Based on the results obtained from the germination experiment, maize cultivar DK 5783 was found to be the most salt tolerant and Apex 836 as the most sensitive cultivar. Also, of six Thi levels used, two levels (100 and 125 mg l^{-1}) were chosen for subsequent studies. In the second experiment (glasshouse experiment), two maize cultivars, DK 5783 (salt tolerant) and Apex 836 (salt sensitive) were subjected to saline regime (100 mM NaCl) and two levels of Thi (100 and 125 mg l^{-1}) applied as foliar spray. Salt stress markedly suppressed shoot and root dry mass, total chlorophylls ("*a*" + "*b*"), leaf water potential and maximum fluorescence yield (*Fv*/*Fm*) in the plants of both maize cultivars, but it increased proline accumulation, leaf osmotic pressure, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations, electrolyte leakage (EL) as well as activities of some key antioxidant enzymes, superoxide dismutase (SOD; EC. 1.15.1.1), peroxidase (POD; EC. 1.11.1.7) and catalase (CAT; EC. 1.11.1.6). Salt-induced reduction in plant growth parameters was higher in the salt-sensitive cultivar, Apex 836, which was found to be associated with relatively increased EL, and MDA and H_2O_2 levels, and decreased activities of the key antioxidant enzymes. Application of Thi as seed soaking or foliar spray partly mitigated the deleterious effects of salinity on plants of both maize cultivars. The most promising effect of Thi on alleviation of adverse effects of salt stress on maize plants was found when it was applied as foliar spray at 100 mg l^{-1}. Thiamin application considerably reduced tissue Na^+ concentration, but improved those of N, P, Ca^{2+} and K^+ in the salt-stressed maize plants. Exogenously applied thiamin-induced growth improvement in maize plants was found to be associated with reduced membrane permeability, MDA and H_2O_2 levels, and altered activities of some key antioxidant enzymes such as CAT, SOD and POD as well as increased photosynthetic pigment concentration under saline regime.

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Abbreviations

CAT	Catalase
SOD	Superoxide dismutase
POD	Peroxidase
Thi	Thiamin
MDA	Malondialdehyde
H_2O_2	Hydrogen peroxide

Introduction

Soil salinity adversely affects both plant growth and productivity by causing high production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide and hydroxyl radical (Ashraf 2009; Foyer et al. 1994; Mittler 2002). All these substances being very reactive are harmful to vital cellular macromolecules such as proteins and lipids (Ashraf 2009; Golldack et al. 2014; Miller et al. 2010; Noctor and Foyer 1998; Noctor et al. 2014). However, to counteract ROS, plants can upregulate their antioxidative defense mechanism by stimulating the activities of key antioxidative enzymes including superoxide dismutases (SOD), catalases (CAT) and peroxidases (POX) (Asada 1999; Ashraf 2009; Sai-Kachout et al. 2013). A variety of plant growth regulators are known to regulate growth and development of most plants under stress conditions including salinity stress. Thiamin is one of such plant growth factors required for growth and differentiation of some plant species (Oertli 1987; Proebsting et al. 1990; Rapala-Kozik et al. 2012). Some in vitro studies give a clue that thiamin can directly act as an antioxidant (Tunc-Ozdemir et al. 2009). Some plant species can synthesize thiamin in roots, while others do not have the ability to produce it in their roots (Mozafar and Oertli 1992), so such plants transport thiamin from shoot to roots (Bonner 1940, 1942). Long ago, Mateikene et al. (1988) reported that plant roots can absorb thiamin and foliar-applied thiamin can move in both acropetal and basipetal directions (Mozafar and Oertli 1992, 1993). It has been thought that under extreme soil or environmental conditions, absorption of this vitamin by the plant roots or leaves could be beneficial for plant growth under such adverse conditions. So, in the present study the effects of thiamin applied as seed soaking or applied through leaves on key plant growth attributes, production of ROS, activities of antioxidant enzymes, and mineral nutrition status were investigated in two maize cultivars with differential salinity tolerance grown under saline conditions.

Materials and methods

Germination experiment

Seeds of nine cultivars of maize were germinated at a constant temperature of 25 ± 1 °C in a thermostatically controlled incubator. Before the initiation of germination experiment, all seed samples were disinfected with sodium hypochlorite solution and then washed with distilled water. They were then soaked for 24 h in distilled water (control), 100 mM NaCl solution or 100 mM NaCl solution supplemented with either of six varying levels, 25, 50, 75, 100, 125 or 150 mg l^{-1} of thiamin. Fifteen seeds of each treatment were distributed in separate Petri plates (15 cm diameter) provided with moist Whatman no. 1 filter paper. Petri plates were moistened each with 10 ml of appropriate treatment solution. To reduce evaporation, Petri plates were covered with parafilm for first 3 days and incubated in the dark.

For recording data for germination percentages, the number of germinated seeds in each Petri plate was recorded every day up to day 7. The data for coleoptile and radicle lengths as well as fresh weight of the seedlings were also recorded.

Germination percentage (GP) was calculated according to the following equation:

$$\text{Gp} = 100(\text{NG}/\text{NT})$$

where

NG = the number of seeds germinated

NT = total number of seeds.

A seed was considered germinated when the radicle emerged from seed coat up to 2 mm. For assessing the rate of germination, time to reach 50 % germination (T_{50}) was calculated according to the following equation:

$$T_{50} = t_i + \left[(N/2 - n_i)\left(t_j - t_i\right)\right]/\left(n_j - n_i\right)$$

where

N = the final number of germinated seeds

n_i, n_j = cumulative number of seeds germinated at periodic counts at times when $n_i < N/2 < n_j$

Radicle and plumule length measurements were also carried out for each treatment. For this purpose, five seeds from each Petri dish were chosen randomly at the end of the experiment.

On day 7, fresh seedling material (200 mg) was homogenized with 70 % (v/v) ethanol and stored in a deep freezer. The alcoholic homogenate was used for the estimation of total soluble sugars and proteins. Trichloroacetic acid (20 %) was used to precipitate the proteins in the homogenate, and sodium hydroxide solution (1 %) was added to the precipitate to dissolve it. The concentration of soluble proteins was quantified using the Lowry et al. (1951) method.

Soluble sugar content was determined by treating 0.1 ml of the alcoholic extract with 3 ml freshly prepared anthrone solution (2,000 mg anthrone + 100 ml 72 % H_2SO_4). It was then placed in a boiling water bath for 10 min as described by Irigoyen et al. (1992). After cooling, the absorbance was read at 620 nm on a UV–visible spectrophotometer.

Glasshouse experiment

Based on the results of the germination experiment, two maize cultivars, DK5783 and Apex 836, differing in salinity tolerance and two most effective thiamin doses were used in this experiment. The same doses of thiamin as used for seed soaking pretreatment were also applied foliarly.

Plant culture and treatments

The experiment was conducted in a glasshouse at the Research Station of the Agriculture Faculty, University of Harran (Turkey) during May and June 2013 with maize (*Zea mays* L. cvs. DK5783 and Apex 836). Five seeds were sown in each pot containing air-dried soil (10 kg in each pot). The texture of the soil used was loamy clay; pH (1:2.5 water, v:v) was 7.3, EC 0.45 dS/m, K 1.40 g/kg, and N 1.25 g/kg. Nitrogen, P_2O_5 and K_2O were mixed into the soil at the rates of 100, 50 and 120 mg/kg as granular urea triple superphosphate and potassium sulfate, respectively.

After germination, the seedlings were thinned to three in each pot, and then placed in a glasshouse for further 35 days at 27 ± 2 °C with mean daytime relative humidity 60–70 %. Before the initiation of the salt treatments, plants were allowed to grow for further 7 days so as to get them established well. The experiment layout was a randomized complete block design with three replicates. Each replicate included three pots (i.e., nine pots per treatment). After determining various physiological parameters of plants of all three replicates, all plants were harvested to determine dry weight and then mineral content.

The volume of water applied every day to the root zone of plants in each container ranged from 50 to 500 mL depending on the size of plants with time.

The two salt treatments applied via irrigation water were: control (no NaCl) and 100 mM of NaCl. Two levels of thiamin (100 and 125 mg l^{-1}) were applied 10 days after seed germination as seed soaking or foliar. Before germination of seeds, they were disinfected with sodium hypochlorite solution (1 % v/v) and then washed with distilled water. For pretreatment of thiamin as seed soaking, the seeds were soaked for 24 h either in 100 or 125 mg l^{-1} of thiamin solution. Plants were sprayed once a week with thiamin solution (50 ml/pot) prepared in 0.01 % tween-20 ($C_{58}H_{114}O_{26}$), a surfactant. The spray was started 10 days after germination and continued up to day 35. Salt stress was maintained by adding 5.85 g NaCl kg^{-1} to the soil via irrigation water prior to planting. Addition of 5.85 g kg^{-1} NaCl to the soil brought the salt level to 100 mM. The EC value of the soil was checked weekly till the termination of the experiment.

At the end of the experiment, fresh and dry weights, inorganic nutrients, chlorophyll content, electrolyte leakage (EL), leaf water potential, leaf osmotic pressure, photosynthetic capacity, activities of antioxidant enzymes, and H_2O_2 and malondialdehyde (MDA) levels were determined.

Chlorophyll determination

Fully expanded youngest leaves were used to extract chlorophyll content. One gram of leaf sample was ground in 90 % acetone solution. The absorbance of the supernatant was measured with a UV/Visible spectrophotometer (Shimadzu UV-1201 V, Japan) and chlorophyll concentrations calculated using the formulae given by Strain and Svec (1966).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was determined in the leaves previously dark- and light-adapted using a portable chlorophyll fluorometer (Photosynthesis Yield Analyzer Mini-PAM, Walz, Germany). The data on the following fluorescence parameters were measured: minimum fluorescence (*F*o), maximal fluorescence (*F*m), and variable fluorescence (*F*v). From these data, maximum quantum efficiency of PSII was calculated as *F*v/*F*m.

Free Proline content

Free proline in fresh leaves was determined by the method described by Bates et al. (1973). Fresh leaf material (0.5 g) was triturated in 10 ml of 3 % aqueous sulfosalicylic acid. An aliquot (2 ml) of the filtrate was reacted with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid. After heating the mixture for 1 h at 100 °C in a water bath, the mixture was extracted with 4 ml toluene. The chromophore containing toluene was aspirated, cooled down to room temperature, and the optical density read at 520 nm.

Leaf osmolality

To determine osmotic pressure, leaves had been shock frozen in liquid nitrogen and stored at −80 °C for 72 h. Thereafter, the samples were thawed, and extracted their sap using a syringe. The sap was centrifuged for 5 min at 5,000*g*, and the osmolality (osmol kg^{-1}) of the supernatant determined using a Cryo-osmometer (Osmomat 030, Ganotec).

Leaf water potential

For leaf water potential measurement, youngest leaf (mainly 3rd leaf from top) was detached from each plant at 8.00 a.m. and its water potential measured using a pressure chamber (PMS model 600, USA).

Electrolyte leakage

For determining EL, fresh leaf samples (200 mg) were cut into small pieces about 5 mm size and placed them in test tubes each containing 10 ml distilled deionized water. The tubes were covered with plastic caps and then placed in a water bath at 32 °C. After 2 h of incubation in the water bath, the initial electrical conductivity of the medium (EC1) was determined with an electrical conductivity meter. Thereafter, the samples were autoclaved at 121 °C for 20 min to get released all electrolytes. The temperature of the samples was brought down to 25 °C and the final electrical conductivity (EC2) measured. The formula (EL = EC1/EC2 × 100) devised by Dionisio-Sese and Tobita (1998) was used to determine EL.

Soluble protein content

The Bradford (1976) protocol was used to quantify soluble protein content in the enzyme extracts using Bovine Serum Albumin V as a standard.

Antioxidant enzyme assays

Fresh leaf samples (each 0.5 g) were ground in 50 mM sodium phosphate buffer (pH 7.0) containing 1 % soluble polyvinylpyrrolidine. After centrifugating the extract for 15 min at 20,000 *g* at 4 °C, it was used for assays of the activities of antioxidant enzymes, CAT, POD and SOD.

The activity of catalase was appraised following Kraus and Fletcher (1994) by determining the consumption of H_2O_2 at 405 nm. The SOD activity was appraised following Beauchamp and Fridovich (1971) based on the ability of the enzyme to suppress the photochemical reduction of NBT (one SOD unit was considered as the quantity of the enzyme used to inhibit cytochrome C reduction by 50 %).

The activity of POD was measured following Chance and Maehly (1955) by adding 100 µl of the tissue extract to 3 ml of assay solution which contained 13 mM guaiacol, 5 mM H_2O_2 and 50 mM Na-phosphate (pH 6.5). The POD activity was appraised as change in absorbance at 470 nm min^{-1} mg^{-1} of protein. The increase in optical density at 470 nm was measured for 3 min and the activity expressed as ΔA_{470}/mg protein/min.

Nutrient analysis and dry weight measurement

The plants were dried in an oven up to constant dry weight and then dry weights of all samples measured. The analysis of inorganic nutrients was conducted using dry plant samples. Total *N* was determined using the Kjeldahl method. For the analysis of other nutrients, the dried and ground samples were washed in a muffle furnace at 550 °C for 6 h. The white ash was dissolved in 5 mL of 2 M hot HCl, and made the final volume to 50 mL with distilled water. Sodium (Na), Ca, and K were analyzed using an ICP (Chapman and Pratt 1982) and *P* following the vanadate–molybdate method.

Determination of lipid peroxidation and hydrogen peroxide

Lipid peroxidation in the leaf samples was appraised by measuring MDA content, a product of lipid peroxidation, following Cakmak and Horst (1991) with some modifications as suggested by Weisany et al. (2012).

Hydrogen peroxide in leaf samples was quantified following Loreto and Velikova (2001). A leaf sample (0.5 g) was ground well in 3 mL of 1 % (w/v) trichloroacetic acid (TCA) and then the extract centrifuged at 10,000 rpm at 4 °C for 10 min. Then an aliquot of 0.75 mL of the supernatant was added to 0.75 mL of 10 mM K-phosphate buffer (pH 7.0) and 1.5 mL of 1 M KI. Its absorbance was read at 390 nm. The concentration of H_2O_2 was calculated from a standard curve plotted using varying levels of H_2O_2 ranging from 100 to 1,000 µmol mL^{-1}. H_2O_2 concentration was expressed as µmol g^{-1}DW.

Statistical analysis

Analysis of variance (ANOVA) of data for all attributes was worked out using the statistical package SAS version 9.1 (SAS Institute Inc., NC, USA). Multivariate analysis of variance (MANOVA) was performed using the SAS GLM procedure to examine differences between the two cultivars and different treatments. The data were considered significant if values were higher than *F* values at $P \leq 0.05$.

Results

Germination experiment

Germination percentage (%)

Salinity stress markedly reduced GP in all cultivars, but considerable variation was observed among the cultivars.

Table 1 Seed germination percentages (%) and time to 50 % seed germination (days) of different maize cultivars germinated in salt and salt with thiamin (mg l^{-1}) applied as seed soaking

Cultivars	C	S	S + Thi 25	S + Thi 50	S + Th 75	S + Thi 100	S + Thi 125	S + Thi 150
Seed germination percentages								
Helen	58ab	45d	48cd	50c	55b	60a	62a	60a
DK 5783	88a	76c	74c	78bc	82b	83ab	83ab	81b
Apex 836	62a	45d	44d	46cd	48bcd	51b	52b	50bc
AYB 936	64a	48c	49c	47c	48c	52bc	54b	52bc
NS 540	65a	46c	48bc	46c	48bc	51b	52b	52b
DK 678	83a	71c	70c	73bc	74bc	74bc	78b	78b
P34N24	80a	68c	67c	67c	69c	73bc	76b	72bc
GW Konsor	81a	71c	70c	71c	72bc	75bc	77b	74bc
NS 640	80a	68c	69c	69c	72bc	73bc	74b	74b
Time to 50 % seed germination								
Helen	6.5c	8.2a	8.2a	7.0b	7.0b	6.4c	6.3c	6.3c
DK 5783	6.0b	6.3ab	6.5a	6.3ab	6.0b	6.0b	6.0b	6.0b
Apex 836	6.4d	7.6ab	7.8a	7.4b	7.4b	7.0c	7.0c	7.0c
AYB 936	6.3b	7.3a	7.3a	7.3a	7.3a	7.0a	7.0a	7.0a
NS 540	6.4c	7.8a	7.4ab	7.8a	7.5a	7.0b	7.0b	7.0b
DK 678	6.2a	6.5a	6.4a	6.5a	6.5a	6.5a	6.3a	6.3a
P34N24	6.3a	6.6a	6.6a	6.6a	6.5a	6.4a	6.4a	6.5a
GW Konsor	6.3a	6.6a	6.6a	6.6a	6.6a	6.4a	6.4a	6.5a
NS 640	6.3a	6.7a	6.7a	6.7a	6.6a	6.6a	6.6a	6.5a

Within each line, means with different letters are significantly different ($P \leq 0.05$)

Thi Thiamin, *C* control, *S* 100 mM NaCl

GP in cv. DK 5783 was less affected, whereas that in cv. Apex 836 B-2 most affected by salinity stress. Thiamin application as seed soaking partially increased the GP of the maize cultivars, however, the two thiamin doses (100 and 125 mg l^{-1}) were the most effective in mitigating the adverse effects of salinity on GP (Table 1).

Time to 50 % germination (T50 %G)

Salinity stress delayed seed germination of all maize cultivars, however, its effect on cv. Apex 836 was more detrimental, whereas the reverse was true for cv. DK 5783. Thiamin application, especially at doses of 100 and 125 mg l^{-1} was most effective in reducing the time to 50 % germination from 6.3 to 6.0 days in cv. DK 5783 and from 7.6 to 7.0 days in cv. Apex 836 (Table 1).

Fresh weight and lengths of plumules and radicles

Salinity stress reduced both fresh weight of hypocotyls and lengths of both plumules and radicles in all tested cultivars to a varying extent. Reductions in fresh weight and lengths of both plumules and radicles were highest in cv. Apex 836 and lowest in cv. DK 5783. However, thiamin applications partially improved fresh weight and lengths of plumules and radicles in all cultivars, but such thiamin-induced improvements were more promising at its 100 and 125 mg l^{-1} levels (Table 2).

Total soluble sugars and proteins

Salinity stress increased total soluble sugars but reduced total soluble proteins in the plumules of all cultivars tested to a variable extent. Of the cultivars, the highest soluble sugar and protein contents were observed in cv. DK 5783, but lowest in cv. Apex 836 (Table 3). Thiamin applications partially reduced soluble sugars and increased soluble protein content in all cultivars, but their values were still far from the control values. In most cases, the highest doses of thiamin were most effective in reducing soluble sugars and increasing protein contents.

Glasshouse experiment

Based on the results of the germination experiment, of nine cultivars, cv. DK 57 83 (salt tolerant) and cv. Apex 836 (salt sensitive) and of six thiamin doses, two (100 and 125 mg l^{-1}) were selected for the glasshouse experiment.

Some key growth parameters

Fresh and dry weights of both cultivars were reduced significantly by salinity stress, but the reductions were higher in cv. Apex 836 than those in cv. DK 5783. Pre-sowing seed treatment and foliar application of thiamin improved fresh and dry weights in both maize cultivars, however, the latter mode of application was more effective than the

Table 2 Lengths of plumules plus radicles (mm) and fresh weight (mg/germinated seed) of seeds of different maize cultivars germinated in salt and salt with thiamin (mg l^{-1}) applied as seed soaking

Cultivars	C	S	S + Thi 25	S + Thi 50	S + Thi 75	S + Thi 100	S + Thi 125	S + Thi 150
Lengths of plumules plus radicles								
Helen	24a	13c	12c	16bc	15bc	18b	19b	18b
DK 5783	36a	28c	28c	29bc	29bc	32abc	33ab	32abc
Apex 836	22a	14c	15bc	15bc	16bc	18b	20a	20a
AYB936	27a	19d	17d	18d	20cd	24bc	25b	23bc
NS 540	28a	20d	20d	20d	21d	25bc	25bc	23cd
DK 678	34a	25c	25c	26bc	27bc	26bc	28bc	29b
P34N24	33a	25c	26c	26c	27bc	29bc	30ab	29bc
GW Konsor	31a	25c	26c	26c	27bc	28abc	30ab	30ab
NS640	30a	23b	23b	24b	25b	25b	26b	25b
Fresh weight of seeds								
Helen	352a	262c	271c	284bc	282bc	294b	302b	301b
DK 5783	445a	402d	415c	412cd	414c	425b	429b	420bc
Apex 836	305a	210e	220d	220d	224d	235c	250b	230cd
AYB 936	312a	225de	230d	220e	224de	248c	259b	232d
NS 540	314a	229d	231d	232d	234d	249c	263b	243c
DK 678	441a	392d	396cd	395cd	398cd	406bc	416b	405bc
P34N24	438a	389b	391b	395b	394b	396b	398b	395b
GW Konsor	430a	380c	382c	384c	384c	397b	398b	395b
NS 640	428a	375d	375d	379d	385cd	386cd	395b	387bc

Within each line, means with different letters are significantly different ($P \leq 0.05$)

Thi Thiamin, *C* control, *S* 100 mM NaCl

Table 3 Total soluble sugars (mg g^{-1} Fw) and soluble protein content (mg g^{-1} dw) in hypocotyls and of seeds of different maize cultivars germinated in salt and salt with thiamin (mg l^{-1}) applied as seed soaking

Cultivars	C	S	S + Thi 25	S + Thi 50	S + Thi 75	S + Thi 100	S + Thi 125	S + Thi 150
Total soluble sugars								
Helen	0.89d	1.89a	1.79b	1.76b	1.75b	1.56c	1.52c	1.59c
DK 5783	0.84d	2.85a	2.80a	2.76ab	2.70b	2.68b	2.50c	2.45c
Apex 836	0.78d	1.52a	1.50ab	1.45ab	1.44b	1.32c	1.32c	1.35c
AYB 936	0.89d	1.86a	1.78b	1.80ab	1.76b	1.68c	1.66c	1.65c
NS 540	0.86d	1.82a	1.73b	1.82a	1.72b	1.64c	1.63c	1.63c
DK 678	0.84d	1.81a	1.78a	1.72b	1.74b	1.68bc	1.66c	1.69bc
P34N24	0.82e	1.82a	1.76ab	1.74bc	1.73bc	1.68cb	1.60d	1.62d
G W Konsor	0.86f	1.95a	1.86b	1.81bc	1.76c	1.62d	1.54e	1.62d
NS 640	0.82f	1.79a	1.75ab	1.74ab	1.72b	1.63c	1.56d	1.62c
Soluble protein content								
Helen	12.5a	8.4c	8.6c	8.6c	8.2c	9.6b	9.8b	9.4b
DK 5783	12.9a	9.6b	9.8b	9.8b	9.6b	10.1b	10.2b	10.0b
Apex 836	12.6a	7.8c	8.0c	8.2bc	8.2bc	8.6b	8.8b	8.6b
AYB 936	12.5a	8.6c	8.7c	8.8c	8.8c	9.2bc	9.4b	9.1bc
NS 540	12.4a	8.5c	8.6c	8.6c	8.8c	9.4b	9.5b	9.2bc
DK 678	12.6a	8.8c	8.9c	8.8c	8.9c	9.2bc	9.5b	9.5b
P34N24	12.4a	8.5d	8.8cd	8.8cd	8.9cd	9.2bc	9.3bc	9.6b
GWKonsor	12.5a	8.8c	8.9c	8.8c	9.2bc	9.1bc	9.5b	9.3b
NS 640	12.4a	8.6c	8.9c	9.0c	9.1bc	9.3bc	9.5b	9.4bc

Within each line, means with different letters are significantly different ($P \leq 0.05$)

Thi Thiamin, *C* control, *S* 100 mM NaCl

former (Table 4). MANOVA revealed significant differences between the two cultivars and different treatments in terms of fresh weight and dry weight at $P \leq 0.05$ (Table 4).

Salinity stress reduced maximum fluorescence yield (*Fv*/*Fm*) and total chlorophyll content, but increased membrane permeability (MP) of both cultivars. However, cv. Apex 836 was more affected by salinity than cv. DK

Table 4 Fresh and dry weights, maximum fluorescence yield (*Fv*/*FM*), membrane permeability (MP), and total chlorophyll (mg kg^{-1} Fw) of plants of different maize cultivars grown in salt with or without different levels of thiamin (mg l^{-1}) applied through different modes

Cultivars	Treatments	FW (g/p)	DW (g/p)	*Fv*/*FM*	MP (%)	Chl.
DK 5783	C	16.3a	1.86a	0.62a	16c	1256a
	S	9.7d	1.11d	0.58c	24a	1056d
	sThi 100	12.4bc	1.26c	0.60b	22a	1120c
	sThi 125	12.1bc	1.21c	0.60b	21b	1132c
	fThi 100	13.6b	1.42b	0.61ab	18bc	1205b
	fThi 125	10.3cd	1.10d	0.59c	19b	1189b
Apex 836	C	12.3a	1.29a	0.60a	19d	1198a
	S	6.7c	0.71d	0.54d	29a	1005d
	sThi 100	7.1c	0.79c	0.56c	25bc	1075c
	sThi 125	7.3c	0.82c	0.56c	26ab	1070c
	fThi 100	8.2b	0.90b	0.59a	23c	1105b
	fThi 125	8.1b	0.93b	0.58b	24bc	1056c
Cvs × treatments		*	*	*	*	ns

Means marked with different letters in the same column within a same cultivar indicate significant difference between treatments at $P \leq 0.05$

Thi Thiamin, *C* control, *S* 100 mM NaCl, *s* seed application, *f* foliar application (mg l^{-1})

MANOVA: * $P \leq 0.05$

5783. Both seed and foliar applications of thiamin improved these key parameters. Overall, foliar application of thiamin at 100 mg l^{-1} was found to be more effective than other thiamin treatments used for both cultivars (Table 4). The results of MANOVA analysis show that there were significant differences between the cultivars and treatments for *Fv*/*Fm* and MP, but not for total chlorophyll content at $P \leq 0.05$ (Table 4).

Leaf water potential (Ψl) was reduced by 100 mM NaCl treatment, but salinity stress increased leaf osmolality (LO) and proline (Pro) content in both cultivars. Salinity stress was more detrimental on leaf water potential of the salt-sensitive cultivar, Apex 836. Salinity stress also resulted in elevating leaf osmolality and proline content in the salt-sensitive cultivar (Table 5). MANOVA revealed that significant differences existed between the cultivars and treatments for Ψl, LO and Pro ($P \leq 0.05$) as shown in Table 5.

Both seed and foliar applications of thiamin improved leaf water potential and reduced leaf osmolality and proline content to levels, being significantly higher than those at control conditions. In most cases, foliar application of thiamin was more effective.

Mineral nutrients

As expected, Na^+ concentrations were higher in the salt-stressed plants of both maize cultivars. The salt-sensitive cultivar Apex 836 had higher Na^+ concentration than the salt tolerant cultivar DK5783. Both modes of thiamin application reduced Na^+ content but it was still much higher than that under control conditions. Furthermore, salinity reduced N, P, Ca and K concentrations in the leaves of both cultivars, but these reductions were higher in the salt-sensitive cultivar Apex 836. Seed and foliar application of thiamin reduced leaf Na^+, but increased the levels of other elements analyzed. Foliar application of thiamin seemed to be more effective in improving nutrient elements in the leaves of both cultivars (Table 6). Significant differences were obtained between the cultivars and treatments by MANOVA at $P \leq 0.05$ for all nutrients analyzed (Table 6).

Antioxidant enzymes and ROS

Salt stress increased the activities of SOD, CAT and POX in both cultivars and these improvements were higher in the salt tolerant cultivar, DK 5783, than those in the salt-sensitive cv. Apex 836. Seed and foliar applications of thiamin reduced the activities of all antioxidant enzymes tested, but foliar application of thiamin at 100 mg l^{-1} being more effective in reducing the activities of these enzymes (Table 7). There were significant differences between the cultivars and among the treatments for SOD and CAT, but not for POX according to MANOVA at $P \leq 0.05$ (Table 7).

Hydrogen peroxide and MDA contents also increased in the leaves of both cultivars grown at saline regime. Both H_2O_2 and MDA contents were higher in the salt-sensitive cultivar grown under saline regime (Table 7). Thiamin applied via seed or leaves reduced H_2O_2 and MDA

contents in the leaves of both maize cultivars. Foliar application of thiamin at 100 mg l^{-1} was more effective in reducing the ROS. MANOVA showed significant differences between the cultivars and among the treatments for both H_2O_2 and MDA at $P \leq 0.05$ (Table 7).

Table 5 Leaf water potential (Ψl: MPa), leaf osmolality (LO, Osmol kg^{-1}) and free proline content (Pro, μmol g^{-1}) in plants of different maize cultivars grown in salt with or without different levels of thiamin (mg l^{-1}) applied through different modes

Cultivars	Treatments	Ψl	LO	Pro
DK 5783	C	−0.35a	0.045e	1.11d
	S	−1.45e	0.126a	2.86a
	sThi 100	−1.33d	0.112b	2.39b
	sThi 125	−1.30cd	0.111b	2.45b
	fThi 100	−1.03b	0.095d	2.29c
	fThi 125	−1.20c	0.102c	2.31c
Apex 836	C	−0.32a	0.042d	1.13c
	S	−1.58d	0.136a	2.66a
	sThi 100	−1.41c	0.125b	2.41b
	sThi 125	−1.36c	0.126b	2.42b
	fThi 100	−1.12b	0.105c	2.41b
	fThi 125	−1.34c	0.112c	2.46b
Cvs × treatments		*	*	*

Means marked with different letters within a same column of a cultivar indicate significant difference between treatments at $P \leq 0.05$

Thi Thiamin, *C* control, *S* 100 mM NaCl, *s* seed application, *f* foliar application (mg l^{-1})

MANOVA: *ns* not significant; * $P \leq 0.05$

Discussion

Soil salinity or saline water irrigation is generally a principal cause of inhibition in seed germination and reduction in biomass and crop production (Ashraf et al. 2008). Better seed germination and subsequent seedling establishment under salt stress is believed to impart enhanced stress tolerance at later growth stages so as to produce better crop growth and productivity (Ashraf et al. 2008; Ashraf and McNeilly 2004; Francois 1994). In the present study, a marked inhibitory effect of salt stress on seed germination percentage, speed of seed germination and seedling biomass production of all maize cultivars was observed. Of nine maize cultivars, cv. DK 5783 was found to be the most salt tolerant while cv. Apex 836 the most salt sensitive. Inhibition in seed germination and seedling growth of maize cultivars might be the result of salt-induced alteration in key physiological and biochemical processes such as plant hormonal concentrations, and activities of metabolic enzymes responsible for utilization of seed food reserves during germination (Ashraf and Foolad 2005). Seed priming with 100 or 125 mg l^{-1} of thiamin in nine maize cultivars resulted in increased seed germination percentage, speed of seed germination, and growth of seedlings, whereas lower concentration of exogenously applied thiamin was less effective in mitigating the adverse effects of salt stress on maize plant growth, particularly at the initial growth stages. Since seeds have low water contents and low levels of antioxidants under saline conditions, they are poorly protected from stress-induced oxidative stress at the seed germination stage. It is likely

Table 6 Sodium, nitrogen, phosphorus, calcium and potassium concentrations (mmol kg^{-1}) of different cultivars of maize grown in salt with or without different levels of thiamin (mg l^{-1}) applied through different modes

Cultivars	Treatments	Na	N	P	Ca	K
DK 5783	C	34e	1,150a	66a	172a	355a
	S	325a	885e	34c	110d	254d
	sThi 100	295b	955d	44b	120c	260cd
	sThi 125	274c	980c	46b	122c	265c
	fThi 100	232d	980c	51b	136b	314b
	fThi 125	246d	1,018b	48b	129bc	304b
Apex 836	C	31c	1,125a	62a	165a	341a
	S	395a	840e	29c	95c	221e
	sThi 100	325b	923d	32c	101c	231d
	sThi 125	332b	934d	31c	112c	241c
	fThi 100	324b	1,025c	42b	125b	272b
	fThi 125	335b	1,059b	44b	122b	265b
Cvs × treatments		*	*	*	*	*

Means marked with different letters within a same column of a cultivar indicate significant difference between treatments at $P \leq 0.05$

Thi Thiamin, *C* control, *S* 100 mM NaCl, *s* seed application, *f* foliar application (mg l^{-1})

MANOVA: * $P \leq 0.05$

Table 7 Superoxide dismutase (SOD: unit mg protein^{-1} min^{-1}), catalase (CAT: unit ×100/mg protein), peroxidase (POD: ΔA_{470}/min/mg protein), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations in leaves of different cultivars of maize grown in salt with or without different levels of thiamin (mg l^{-1}) applied through different modes

Cultivars	Treatments	SOD	CAT	POD	H_2O_2 (μmol g^{-1} DW)	MDA (nmol g^{-1} FW)
DK 5783	C	46e	1.30e	8.18c	1.15d	1.35d
	S	174a	2.91a	36.12a	6.52a	10.25a
	sThi 100	134b	2.42b	22.23b	4.32b	6.26b
	sThi 125	132b	2.34b	25.35b	4.26b	6.77b
	fThi 100	98d	1.99d	22.25b	3.25c	4.63c
	fThi 125	114c	2.21c	23.34b	4.52b	6.88b
Apex 836	C	48e	1.35e	8.96c	1.26d	1.54d
	S	155a	2.69a	35.25a	8.65a	13.25a
	sThi 100	116b	2.22b	22.36b	6.36b	9.56b
	sThi 125	106c	2.15b	24.36b	6.39b	9.65b
	fThi 100	95d	1.85d	19.63b	5.36c	7.23c
	fThi 125	104c	1.96c	20.36b	6.25b	9.63b
Cvs × treatments		*	*	ns	*	*

Means marked with different letters within a same column of a cultivar indicate significant difference between treatments at $P \leq 0.05$

Thi Thiamin, *C* control, *S* 100 mM NaCl, *s* seed application, *f* foliar application (mg l^{-1})

that during early seedling development/seed germination stage, priming with water soluble antioxidants like thiamin may have an important protective function (Ashraf and Foolad 2005; Plaut et al. 2013). Recently, it has been shown that thiamin application induces oxidative stress tolerance in plants (Ahn et al. 2005, 2007; Tuna et al. 2013; Tunc-Ozdemir et al. 2009). The mitigating effect of thiamin on maize cultivars might have been due to its role as coenzyme in various metabolic pathways such as sugar and protein metabolism (Goyer 2010). In the present study, soluble sugar and soluble protein contents in the hypocotyls of maize cultivars decreased, whereas upon thiamin application to maize plants these biochemicals increased. These results suggest that thiamin application played an effective role in regulation of carbon metabolism and protein synthesis as a functional coenzyme in the metabolic pathways of these biochemicals. Accumulation of soluble sugars in response to thiamin application in salt-stressed maize plants may be an important adaptive response to salt stress (Ashraf and Harris 2004). Thus, it is likely that improvement in seed germination in all maize cultivars due to seed soaking treatment with 100 or 125 mg l^{-1} might have been due to thiamin-induced protective effects on cellular carbon and nitrogen metabolisms against salt-induced oxidative stress in germinating seeds. Moreover, such an ameliorative effect of thiamin was more on cv. DK 5783 than that on cv. Apex 836. Quite similar to this, seed soaking treatment with other non-enzymatic antioxidant compounds had differential effect in wheat (Athar et al. 2008), canola (Athar et al. 2009), and maize (Ali et al. 2007).

Mode of application of chemicals to plants is believed to have differential mitigating effects on different crops (Athar et al. 2009; Khan et al. 2006; Plaut et al. 2013), so a detailed study on comparison of modes of thiamin application as seed soaking or foliar application was also conducted at the vegetative growth stage in the present study. Seed soaking or foliar application of 100 or 125 mg l^{-1} of thiamin improved the growth of both maize cultivars under saline conditions. However, foliar application of 100 mg l^{-1} thiamin was found to be most effective in mitigating the adverse effects of salt stress on the salt tolerant and salt-sensitive maize cultivars. These results are similar to those of Sayed and Gadallah (2002) in which 5 or 10 mg l^{-1} thiamin application as foliar spray or its addition to the rooting medium ameliorated the adverse effects of salt stress on sunflower plants. Similarly, while assessing the comparative effect of foliar application of three different non-enzymatic antioxidants in alleviating the adverse effects of salt stress on maize. Tuna et al. (2013) have reported that 100 mg l^{-1} thiamin application as foliar spray improved the growth and productivity of maize under saline conditions. Difference in use of effective dose of thiamin in these reports and that in the present study might have been due to time interval of foliar application of thiamin. For example, Sayed and Gadallah (2002) applied 5 or 10 mg l^{-1} thiamin as foliar application after every 2 days, whereas in the present study foliar application of thiamin was applied on weekly basis.

A question arises as to how thiamin mitigated the adverse effects of salt stress on maize plants. In a previous study, Sayed and Gadallah (2002) found that thiamine-

induced increase in growth was associated with lower membrane EL, improved chlorophyll content, and increased amount of soluble sugars and total free amino acids. Ashraf and Akram (2009) reviewed that under stress conditions, over-reduction of ferredoxin by photosynthetic electron transport chain causes the generation of ROS via Mehler Reaction and plants with their innate detoxifying system including non-enzymatic antioxidants are better able to scavenge these ROS. This is the rationale of using exogenous application of non-enzymatic antioxidants to increase antioxidant potential and hence salt tolerance of plants (Plaut et al. 2013). In the present study, mitigating effect of exogenous application of thiamin was found to be associated with reduced generation of ROS in plants of both maize cultivars, e.g. lower accumulation of H_2O_2 and MDA contents in thiamin-treated maize plants was observed (Table 7). This argument can be further supported by the fact that salt stress increased the activities of all antioxidant enzymes (SOD, POD and CAT), whereas thiamin treatment reduced the activities of these enzymes in both maize cultivars (Table 7). These results are similar to those of Tuna et al. (2013) in which exogenous application of non-enzymatic antioxidants reduced the activities of antioxidant enzymes in salt-stressed maize plants. In view of these findings, it is suggested that increase in antioxidant enzyme activities under salt stress is an additional burden on metabolism of maize plants and exogenous thiamin application reduced this metabolic burden in salt-stressed plants of both maize cultivars. This statement is further supported by the findings of Ahn et al. (2005, 2007) in which thiamin-treated cucumber, tobacco, rice and *Arabidopsis* showed greater multiple stress resistance due to thiamine-induced reduction in metabolic cost for constitutive expression of certain genes. In maize seedlings, tolerance to salt stress was found to be associated with scavenging of ROS and an increase in thiamin content (Rapala-Kozik et al. 2008). Rapala-Kozik et al. (2012) suggested that thiamin mediates oxidative stress tolerance via salicylic acid and Ca^{2+}-related signaling pathways. In another study, it was shown that thiamine pretreatment modulates the cellular redox status via ascorbate peroxidase and NADPH-oxidase-dependent ROS signaling, thereby protecting *Arabidopsis thaliana* against *Sclerotinia* (Zhou et al. 2013). In view of our results and these reports, it is suggested that thiamin application modulates antioxidant potential of maize plants thereby improving salt tolerance. Overall, thiamin application as seed soaking was less effective as compared to foliar spray. Moreover, thiamin application was more effective in alleviating the adverse effects of salt stress on the salt tolerant maize cv. DK 5783. Exogenously applied thiamin has been reported to be effective in improving plant growth due to upregulation of different physio-biochemical processes (Al-Hakimi and Hamada 2001; Sayed and Gadallah 2002; Goyer, 2010; Tuna et al. 2013). However, role of exogenously applied thiamin in oxidative defense system (enzymatic/non-enzymatic) yet needs to be elucidated. In the present study, antioxidant enzymes such as SOD, POX and CAT decreased due to application of thiamine under saline conditions. Analogous to our results, Tuna et al. (2013) have recently observed that foliar-applied thiamin (100 mg L^{-1}) decreased the activities of SOD and POX, while did not alter PPO in salt-stressed maize plants. In Arabidopsis, Tunc-Ozdemir et al. (2009) showed that exogenous-applied thiamin improved tolerance against oxidative stress by minimizing H_2O_2 content induced by multiple stresses including salinity stress.

Chloroplast is the principal site for generation of ROS under saline conditions and this may result in degradation of thylakoid membranes and photosynthetic pigments (Taiz and Zeiger 2010). In the present study, it was found that thiamin application counteracted the inhibitory effect on chlorophyll contents particularly in cv. DK 5783. The differential effect of thiamin application on maize cultivars could be due to lower H_2O_2 accumulation and MDA in the salt tolerant cv. DK 5783 than that in cv. Apex 836. In higher plants, generation of H_2O_2 and lipid peroxidation of chloroplast membranes under salt stress favor chlorophyll degradation (Hörtensteiner 2013). Enhancement in chlorophyll content in salt-stressed plants of both maize cultivars due to thiamin application can be reasoned to the protective effect of thiamin on organellar membranes. In the present study, membrane permeability was reduced in salt-stressed plants of both maize cultivars due to thiamin application. Membrane permeability is widely used as an indicator of membrane injuries in salt-treated plants and can be used as a potential indicator for salt tolerance (Ashraf and Ali 2008). Moreover, this protective effect of thiamin on membranes was more when 100 mg l^{-1} thiamin applied as a foliar spray to maize plants (Table 4). These results are similar to those of Tuna et al. (2013) in which 100 mg l^{-1} thiamin as a foliar spray improved chlorophyll content and reduced the membrane permeability in maize plants under saline conditions.

All land plants transform solar energy into chemical form of energy via photosynthetic electron transport that can be used for fixation of CO_2 in Calvin cycle. Although it is well documented that thiamin has a major role in regulating oxidative pentose phosphate pathway and scavenging H_2O_2 around photosystem I (PSI) (Goyer 2010), it is not known whether it has a role in structural stability of photosystem II (PSII) or its activity for conversion of solar energy into biochemical energy. In the present study, thiamin application mitigated the adverse effects of salt stress on PSII activity (F_v/F_m). In view of available information, it can be suggested that thiamin might have a protective

effect on D1 protein of PSII thereby resulting in enhanced PSII activity in both maize cultivars under saline conditions as has earlier been observed in wheat due to ascorbic acid treatment (Athar et al. 2008). Another explanation of greater PSII activity due to thiamin application is that exogenous use of thiamin reduced the osmotic stress by improving leaf water potential and accumulation of proline (Table 5) in the leaves of salt-stressed plants of both maize cultivars, as has been observed in the present study. Similar mitigating effects of thiamin application on leaf water content and proline accumulation were observed in maize (Tuna et al. 2013) and sunflower (Sayed and Gadallah 2002). It is well evidenced that proline acts as a compatible solute and participates in radical detoxification and enzyme protection (Ashraf and Foolad 2007). Earlier, Al-Hakimi and Hamada (2001) found that seed priming with thiamine alleviated the adverse effects of salt stress on wheat seedlings by lowering the accumulation of proline. As discussed earlier, thiamin application scavenges ROS and reduces the metabolic cost for biosynthesis of extra amount of proline. In view of all these reports and the results from the present study, it is suggested that thiamin application improved the plant water status and antioxidant potential by fine-tuning different metabolic pathways thereby resulting in enhanced photosynthetic activity and growth of maize cultivars under saline conditions.

Regulation of ion homeostasis is one of the main corner stones for salt tolerance in plants (Ashraf 2004; Munns and Tester 2008). In the present study, salt stress decreased mineral nutrients such as N, P, K and Ca^{2+}, whereas it increased Na^{+} accumulation in the leaves of salt-stressed maize plants. Exogenously applied thiamin reduced Na^{+} accumulation in the leaves while it improved N, P, K and Ca^{2+}. These results are similar to those of Tuna et al. (2013) in which foliar spray of thiamin was reported to reduce Na^{+} accumulation in leaves and roots, while increased the accumulation of N, P, K and Ca^{2+} in the salt-stressed plants of both maize cultivars. Goyer (2010) reviewed that thiamin applied either as a foliar spray or through the rooting medium is rapidly taken up by the plant with its subsequent transportation to other parts of the plant via xylem pathway. In view of thiamin transport pathway in plants, it is suggested that thiamin might have a role in preferential uptake of K^{+} on Na^{+} at the xylem loading site or simply thiamin might have a role in the maintenance of ion homeostasis. It is well documented that ionic changes reversibly trigger the antioxidant system (Mittler 2002; Yasmeen et al. 2013).

Taken together, exogenous application of thiamin modulated water and ion homeostasis as well as redox balance that resulted in better photosynthetic capacity and growth of both maize cultivars. However, effectiveness of thiamin application in inducing salt tolerance in maize plants depends on their genotypic potential as well. From a crop improvement against salt stress perspective, modulation of thiamin metabolism using its exogenous application may be an innovative way to improve abiotic stress tolerance in crops.

Author contribution statement Cengiz Kaya, Osman Sonmez, Levent Tuna and Tahir Polat worked on all experiments in the project, on data analysis and writing the report. Muhammed Ashraf and Salih Aydemir worked on the writing and editing of the manuscript for publication.

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